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Atrazine and the Hypothalamo-Pituitary-Gonadal Axis in Sexually Maturing Precocial Birds: Studies in Male Japanese Quail

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The herbicide atrazine is a putative endocrine disruptor. The present studies investigated the effects of atrazine in male Japanese quail during sexual maturation. Atrazine was administered for two weeks in the diet or systemically to birds under long photoperiods. Atrazine had no effect on mortality but depressed both feed intake and growth (average daily gain [ADG] in g/day) at dietary concentrations of 1000 ppm. Atrazine in the diet at 10 ppm, but at no other concentrations, increased testes weight and gonadal-somatic-index and decreased the seminiferous tubule diameter-to-testis weight ratio. However, there were no effects on absolute tubule diameter, relative stage of testicular development, or the presence of a lumen. Atrazine in the diet at 1000 ppm increased circulating concentrations of testosterone but this effect was not observed consistently in all studies. Dietary atrazine at 10 ppm increased circulating concentrations of estradiol. Moreover, in one study, atrazine at 1000 ppm in the diet decreased circulating concentrations of luteinizing hormone. Atrazine administered systemically exerted no effect on indices of growth or reproduction. Atrazine did not mimic the effects of either estradiol or tamoxifen in male quail; thus, atrazine did not exhibit overt estrogenic or anti-estrogenic activity. Conversely, atrazine augmented the effects of testosterone and estradiol on testis regression, presumably by increasing the negative-feedback effects of these sex-steroids on follicle stimulating hormone secretion. It is concluded that atrazine up to 1000 ppm in the diet may exert some effects on reproductive development in sexually maturing male birds, but these are inconsistent and modest.

Key Words: **atrazine; male quail; testes; reproductive hormones; endocrine disruptor.**

There is increasing concern that chemicals in the environment may be endocrine disruptors. Endocrine disruptors are defined as exogenous chemicals or mixtures that alter the function of the endocrine system, causing adverse effects at the level of the organism, progeny, population, or subpopulation (USEPA, 1998). These compounds exhibit estrogenic, anti-estrogenic, androgenic, or anti-androgenic activities and/or may affect thyroid, immune, and cognitive function (Rhind, 2002). The extensively used herbicide, atrazine, has come under scrutiny as a putative endocrine disruptor.

Atrazine exhibits reproductive toxicity in male rats. It reduces the activity of 5α-reductase and inhibits binding of 5α-dihydrotestosterone (DHT) to the androgen receptor (Babic-Gojmerac *et al.*, 1989; Kniewald *et al.*, 1995). Furthermore, atrazine reduces serum and testicular concentrations of testosterone and luteinizing hormone (LH) (Stoker *et al.*, 2000; Trentacoste *et al.*, 2001). Atrazine increases serum concentrations of estradiol and estrone and delays the time to preputial separation of the penile urethra in the male rat (Stoker *et al.*, 2000).

There are reports of the effects of atrazine on reproductive organ development, but these observations are not consistent. For example, in Fisher rats, atrazine administered orally at 120 mg/kg increased prostate, seminal vesicle, and pituitary weights in rats (Simic *et al.*, 1994). However, in Sprague-Dawley rats, a similar regimen of atrazine (50–200 mg/kg) decreased ventral prostate and seminal vesicle (Stoker *et al.*, 2000; Trentacoste *et al.*, 2001) and pituitary (Stoker *et al.*, 2000) weights. Furthermore, ip injections of atrazine reduced the weight of the pituitary gland and the ventral prostate (Kniewald *et al.*, 2000). In Wistar rats, doses of atrazine of at least 12.5 mg/kg over long periods of time indirectly induce inflammation of the prostate (Stoker *et al.*, 1999). Furthermore, atrazine decreases the rate of epididymal migration of sperm, induces histopathological changes of the testes, reduces testicular protein concentrations, and reduces total sperm number and motility (Kniewald *et al.*, 2000).

Low concentrations of atrazine have been reported to feminize amphibians (Hayes *et al.*, 2002, 2003). This feminization is unlikely to be caused by direct activation of the estrogen receptor (ER) (Connor *et al.*, 1996) but may be attributed to activation of gonadal aromatase (Hayes *et al.*, 2002; Sanderson *et al.*, 2000). Moreover, plasma concentrations

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of testosterone are reduced by atrazine in exposed frogs (Hayes *et al.*, 2002).

Atrazine exhibits low acute toxicity to birds with a dietary LC_{50} of >5000 ppm (Heath *et al.*, 1972; USEPA, 2002). To date, there is little information describing the effect of atrazine on avian reproduction. The current ecological risk assessment for atrazine established by the United States Environmental Protection Agency (USEPA) reports a dietary no-observed-adverse-effect-concentration (NOAEC) of 225 ppm and a lowest-observed-adverse-effect-concentration (LOAEC) of 675 ppm (USEPA, 2002). At this concentration, birds experience reduced egg production, an increase in defective eggs, and a reduction in embryo viability (northern bobwhite quail and mallard duck) (USEPA, 2002). There are no reports describing the effects of atrazine on reproduction in male birds. In the temperate zone, birds may be exposed to atrazine during the spring field application period. At this time, increases in the natural photoperiod induce sexual maturation of birds (Dawson *et al.*, 2001; Follett *et al.*, 1985). The present studies examine the effects of atrazine on the hypothalamo-pituitary-gonadal (HPG) axis in precocial birds, modeled by the male Japanese quail, during photostimulated gonadal maturation.

MATERIALS AND METHODS

Animals and procedure. All procedures were performed at the Iowa State University Poultry Science Research Center and were approved by the Iowa State University Committee on Animal Care (protocol 7-2-5195). Japanese quail (*Coturnix coturnix Japonica*) eggs were generously provided by Dr. Bernard Wentworth (University of Wisconsin-Madison). The quail were reared from hatch in battery cages under a short daily photoperiod (8L:16D) with free access to feed (Purina Game Bird Chow; Purina Mills LLC, St. Louis, MO) and water. At the ages described, males were separated from females based on plumage, transferred to individual cages, and the treatments initiated. At the start of the treatments, the photoperiod was changed to 16L:8D to induce sexual maturation. All dietary treatments were directly mixed in Purina Game Bird Chow; feed and water were available *ad libitum*. In all studies, the dietary concentrations of atrazine (CAS# 1912-24-9, 99.9% purity; Chem Service, Inc, Westchester, PA) and control hormones are considered nominal.

In each study, male quail were weighed and randomly assigned to treatment groups. The birds were fed dietary treatments or controls *ad libitum* (unless noted) for two weeks. They were then re-weighed and blood samples collected after decapitation. Plasma was separated by centrifugation (2500 × *g*, 10 min, 4°C) and stored at −20°C until analysis. The body was dissected and the liver and testes removed, weighed, and frozen in liquid nitrogen or fixed as described in a subsequent section. To correct for differences in body weight, the liver- and gonadal-somatic indices (liver or gonad weight as a percentage of body weight) were determined.

Experimental treatments. Studies 1 and 2 investigated the effects of high concentrations of dietary atrazine in male quail. In study 1, six-week-old male quail ($n = 12$ per treatment) were administered atrazine at 0, 10, 100, or 1000 ppm in the diet. In study 2, four-week-old quail were administered 0 or 100 ppm atrazine ($n = 11$ per treatment) or 1000 ppm atrazine ($n = 13$) in the diet. In study 1, a positive control of 17β-estradiol (E_2) (Sigma-Aldrich, St. Louis, MO) was administered at 100 ppm ($n = 12$), it being hypothesized that atrazine would act as an estrogen *per se* or increase estrogen biosynthesis. Untreated feed was administered as a negative control.

Study 3 investigated the effect of atrazine on growth and sexual maturation in six-week-old birds with an additional group of pair-fed birds ($n = 7$ per treatment). Birds were assigned to dietary treatment groups of 0 or 1000 ppm atrazine. Pair-fed birds were used to differentiate the effects of reduced feed consumption and those due to exposure to atrazine. Each day, these birds received the average amount of untreated feed as that taken in by atrazine-treated birds on the previous day.

In study 4, atrazine was administered in 0.1 ml of propylene glycol daily at 1 and 10 mg/kg through sc injections in the nape of the neck ($n = 8$ per treatment). For comparison, vehicle (propylene glycol) and estradiol-3-benzoate (1 mg/kg/day) controls were run concurrently ($n = 8$ per control). In study 5, atrazine was administered through Silastic implants (1.47 mm ID, 1.96 mm OD, 1.5 cm length; Dow-Corning, Midland, MI) (Fennell and Scanes, 1992) ($n = 8$ per treatment). Implants were saturated in phosphate-buffered saline overnight, dipped in 70% ethanol, and placed subdermally in the dorsal thoracic region. Through change in weight, it was determined that atrazine implants delivered a dose of approximately 1.42 mg/kg/day. For comparison, implant controls of cholesterol (negative control) and estradiol-3-benzoate were run concurrently ($n = 8$ per control).

Studies 6 and 7 examined the putative anti-estrogenic effects of atrazine on the HPG axis in 4-week old male quail. In study 6, treatments of 100 ppm tamoxifen (an anti-estrogen, Sigma-Aldrich), 100 ppm E_2, 1000 ppm atrazine, and combinations of 100 ppm E_2 + 100 ppm tamoxifen and 100 ppm E_2 + 1000 ppm atrazine were administered in the diet for two weeks ($n = 8$ per treatment group). It was hypothesized that atrazine would suppress the negative-feedback of E_2 on gonadotropin secretion and hence block regression of the testes (as indicated by testes weight). In study 7, Silastic implants (1.47 mm ID, 1.96 mm OD, 1.5 cm length) of testosterone or cholesterol (negative control) were placed subcutaneously in the dorsal thoracic region and the birds assigned to dietary treatments containing 0 or 1000 ppm atrazine ($n = 9$ per treatment group). It was hypothesized that atrazine would suppress negative feedback effects of testosterone, inhibiting the reduction in circulating concentrations of LH and testicular weights (normally reflecting decreased secretion of follicle stimulating hormone [FSH]).

Hormone analysis. Plasma concentrations of estradiol and testosterone were determined in duplicate using an ELISA from DRG International, Inc. (Mountainside, NJ) according to the manufacturer's instructions with charcoal-stripped, spiked quail plasma as standards for comparison. Both systems were validated for use with quail plasma by the following methods: (1) parallelism of standard curves produced using charcoal-stripped quail plasma was verified with the standards supplied with the assay, (2) recoveries in spiked, unstripped quail plasma were determined to be greater than 75% (>75% for estradiol, > 95% for testosterone). Where circulating concentrations were below the detection limit of the assay, values at the lowest standard were assigned for statistical analysis (15.1 pg/ml for estradiol, 62.5 pg/ml for testosterone). For estradiol and testosterone, the intra-assay coefficient of variation (CV) was 4.1 and 0.7%, respectively, with all samples within a study analyzed in one assay. Plasma concentrations of LH were determined in duplicate as described by Yang *et al.* (1997). The intra- and inter-assay CV were 8.3 and 10.9%, respectively.

Histologic and morphometric analysis. In studies 1, 2, 3, and 7, the left testis was fixed in Bouin's solution (Sigma-Aldrich) and transferred to 70% ethanol for storage. All procedures for embedding, sectioning, and staining were carried out by the Iowa State University Veterinary Diagnostic Laboratory (College of Veterinary Medicine, Ames, IA) according to standard protocols. The tissues were embedded in paraffin and sectioned at 4 μm at two different levels within the tissue. The sections were adhered to a glass slide and stained with hematoxylin and eosin.

The stained sections were examined by light microscopy using an Olympus CH-2 compound microscope (Olympus America, Inc., Melville, NY). To obtain general information on the state of development of the testes, the classification system described by Mather and Wilson (1964) was employed. Ten seminiferous tubules representative of the tissue were examined and

classified (two sections per sample, 5 tubules per section) (Follett and Maung, 1978). A description of the classification method is outlined in the caption of Table 6. In addition, tubule diameters were measured with an ocular micrometer and the qualitative presence of a lumen was evaluated. Only tubules directly cross-sectioned (as indicated by a circular shape) were used for analysis. To correct for differences in testis size, tubule diameters were also analyzed as a ratio of the diameter to the average testis weight of the treatment group.

Statistics. All data were analyzed by the MIXED procedure using SAS version 9.0 (SAS Institute, Cary, NC). Before analysis by analysis of variance (ANOVA), all data were analyzed for outliers; data points outside of two standard deviations were removed. After outlier removal, a normal distribution among values was assumed. Data in studies 1 and 2 were analyzed by two-way ANOVA (treatment and study). The indices of ADG, feed intake, liver, and testes weights (and their corresponding somatic indices) and the left-to-right testis weight ratio were found to be different between studies. These data were normalized by converting to their respective % average control and re-analyzed by two-way ANOVA (treatment and study). It was determined that the normalized data for studies 1 and 2 were not different and thus were pooled to increase replications. After two-way ANOVA, absolute hormone concentrations in studies 1 and 2 (estradiol, testosterone, and LH) were not different between studies and were directly pooled. Pooled values were analyzed by one-way ANOVA (treatment) and, where differences were found ($p < 0.05$), least-squared means of the treatments were compared to the negative control by the Dunnett-Hsu *t*-test. Studies 3, 6, and 7 were analyzed individually by one-way ANOVA (treatment). In these studies, the least-squared means were analyzed by the Tukey-Kramer test for all possible comparisons. Studies 4 and 5 were analyzed by one-way ANOVA (treatment). Where differences were found, treatment groups were compared to the respective negative controls (propylene glycol vehicle or cholesterol implant) by the Dunnett-Hsu *t*-test. To analyze differences in daily feed intake, data from study 3 were averaged for each day and the control group and the atrazine-treated group were compared on each day by the Bonferroni test. Due to the logarithmic growth of the testes during the first half of sexual development (after photostimulation) (Follett and Maung, 1978), testes weights were $\log_{10}$ transformed before being analyzed by the above procedures. Histological data from studies 1, 2, 3, and 7 (classification, tubule diameter, tubule:testis weight ratio, and lumen presence) were averaged across an individual sample, analyzed by two-way ANOVA for differences between studies (treatment and study). The studies were not different and thus the data were pooled and reanalyzed by one-way ANOVA (treatment). Where significance was found, the least-squared means were separated by the Dunnett-Hsu *t*-test. In all studies, values are reported as least-squared mean ± pooled SEM (calculated from MSE). Significance was determined at $p < 0.05$.

RESULTS

General Toxicity

In the seven studies, only four instances of mortality were observed with atrazine or the positive and negative controls (in study 1: [1/12 in control group, 1/12 in estradiol group], in study 5: [1/8 in atrazine implant group], in study 6: [1/9 in estradiol + atrazine group]). Thus, concentrations of atrazine up to 1000 ppm in the diet had no effect on mortality in sexually maturing male Japanese quail. However, there were some effects on other aspects of general toxicity as outlined in Table 1. For example, atrazine at 1000 ppm decreased growth and feed intake by 32 and 15%, respectively, versus control ($p < 0.001$). However, atrazine had no effect on liver weights or the liver-somatic index. In contrast, estradiol (100 ppm) reduced growth ($p < 0.05$) and feed intake 21% each, respectively, versus control ($p < 0.001$). Moreover, estradiol increased liver weights and liver-somatic index 50 and 58%, respectively, versus control ($p < 0.001$).

Sexual Development and Circulating Hormones

The effects of atrazine on indices of reproductive development are summarized in Table 2. It was hypothesized that atrazine would act as an estrogen, decreasing testes weight compared to controls. Atrazine up to 1000 ppm had no effect on testes weight or the ratio of the left-to-right testis weight. However, atrazine at 10 ppm increased testes weight 46% ($p < 0.01$) and gonadal-somatic index 49% versus control ($p < 0.01$). No other concentration of atrazine tested affected this parameter. In contrast, estradiol at 100 ppm decreased testes weight and gonadal-somatic index by 92 and 91%, respectively, versus control ($p < 0.001$). Furthermore, estradiol increased the left-to-right testis weight ratio by 879% versus control ($p < 0.001$).

The effects of atrazine on circulating reproductive hormones are outlined in Table 3. Atrazine up to 1000 ppm had no effect

TABLE 1
Effects of Atrazine on Indices of General Toxicity in Photostimulated Male Quail

Treatment	*n*	Average daily gain	Daily feed intake	Liver	Liver-somatic index
Control value	21	1.7 ± 0.11 g	21.8 ± 0.66 g	3.0 ± 0.15 g	2.3 ± 0.10%
			% Mean for control group in each study		
Control	21	100 ± 6	100 ± 2	100 ± 4	100 ± 4
Atrazine					
10 ppm	12	85 ± 8	97 ± 3	101 ± 56	102 ± 5
100 ppm	23	97 ± 6	95 ± 2	108 ± 4	106 ± 4
1000 ppm	22	68 ± 6***	85 ± 2***	95 ± 4	100 ± 4
Estradiol					
100 ppm	11	79 ± 9*	79 ± 3***	150 ± 6***	158 ± 5***

Note. Data within two studies (studies 1 and 2) were converted to their % average control within each study and then pooled for analysis. Values are presented as the least-squared means ± pooled SEM.

Different from control, *$p < 0.05$, ***$p < 0.001$.

TABLE 2
Effects of Atrazine on Indices of Reproductive Development in Photostimulated Male Quail

Treatment	*n*	Testes weight	Gonadal-somatic index	Left-to-right testis ratio
Control value	21	621 ± 45 mg	0.5 ± 0.04%	0.9 ± 0.06
		% Mean for control group in each study		
Control	21	100 ± 10	100 ± 10	100 ± 120
Atrazine				
10 ppm	12	146 ± 14**	149 ± 13**	90 ± 154
100 ppm	23	116 ± 10	114 ± 10	106 ± 117
1000 ppm	22	115 ± 9	121 ± 9	105 ± 114
Estradiol				
100 ppm	11	8 ± 14***	9 ± 14***	879 ± 169***

Note. Data within two studies (studies 1 and 2) were converted to their % average control within each study and then pooled for analysis. Values are presented as the least-squared means ± pooled SEM. N.D = not determined.
Different from control, $^{**}p < 0.01$, $^{***}p < 0.001$.

on circulating concentrations of LH. However, atrazine at 1000 ppm increased circulating concentrations of testosterone 3.0-fold versus control ($p < 0.01$). Moreover, atrazine at 10 ppm increased circulating concentrations of estradiol 3.6-fold versus control ($p < 0.001$). There were no changes in circulating estradiol at the other concentrations of atrazine tested (100 and 1000 ppm).

Atrazine and Feed Restriction

To eliminate confounding effects observed with reduced feed intake, the effects of atrazine administered in the diet were compared using pair-fed birds (Table 4). Similar to the results above (Table 1), atrazine at 1000 ppm reduced growth by 63.6% versus control ($p < 0.05$). However, atrazine had no effect on liver weights or liver-somatic index versus control. In contrast, growth and liver weights (but not liver-somatic index) were reduced by 90.9 and 25.9% in pair-fed birds versus control. While there were no effects of atrazine on total feed intake (Table 4), atrazine decreased feed intake on several days throughout the experiment (Fig. 1). Neither atrazine nor feed restriction in the pair-fed birds had any effect on testes weights, gonadal-somatic index, or circulating concentrations of estradiol and testosterone. Similarly, there were no differences in the measured indices between atrazine-treated and pair-fed birds.

TABLE 3
Effects of Atrazine on Circulating Concentrations of Reproductive Hormones in Photostimulated Male Quail

Treatment	*n*	Testosterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)
Control	21	463 ± 218	56 ± 21	3.4 ± 0.75
Atrazine				
10 ppm	12	806 ± 308	203 ± 30***	3.7 ± 1.05
100 ppm	21	829 ± 208	77 ± 21	4.0 ± 0.74
1000 ppm	22	1388 ± 214**	25 ± 21	3.4 ± 0.75

Note. Values are presented as least-squared means ± pooled SEM. Data within two studies (studies 1 and 2) were pooled for analysis.
Different from control, $^{**}p < 0.01$, $^{***}p < 0.001$.

Systemic Atrazine

To compare the effects of dietary and systemic atrazine, sc injections and Silastic implants were employed. The results of these studies are outlined in Table 5. Atrazine administered at 1 and 10 mg/kg/day through sc injections had no effect on indices of growth or reproductive development (Table 5). In contrast, sc injections of estradiol-3-benzoate decreased both testes weights and gonadal-somatic index by 46.8 and 42.9%, respectively, versus control ($p < 0.05$). However, the estrogen control had no effect on the other parameters analyzed.

Atrazine administered by Silastic implant (~1.42 mg/kg/day) had no effect on indices of growth and reproductive development (Table 5). In contrast, the estrogen control delivered by Silastic implant increased liver weights and liver-somatic index 44.4 and 50.0%, respectively, versus control ($p < 0.001$). Furthermore, estrogens delivered by implant decreased testes weights and gonadal-somatic index by 76.5 and 83.3%, respectively ($p < 0.05$).

Testis Histology and Morphometric Analysis

In studies 1, 2, 3, and 7, the effects of atrazine on development of the left testis were examined using morphometric analyses. The pooled results of these studies are outlined in Table 6. Atrazine and pair-feeding had no effect on the stage of development of the testes (class), lumen size, or seminiferous tubule diameter. However, atrazine at 10 ppm decreased the tubule diameter-to-testis weight ratio 33.3% versus control ($p < 0.05$). No other concentration of atrazine (or pair-feeding) altered this parameter.

Effects of Atrazine in the Presence of Reproductive Steroids

In study 6, the effects of atrazine on growth in the presence of dietary estradiol were examined. The results of this study are outlined in Table 7. As expected, atrazine decreased growth and feed intake 31.6 and 14.1%, respectively, versus control ($p < 0.05$). Addition of estradiol to the diet decreased feed intake 15.6% ($p < 0.05$) and tended to decrease growth versus control ($p = 0.08$). Furthermore, estradiol increased liver-somatic index, but not absolute liver weights, by 26.9% versus control ($p < 0.05$). In contrast, concurrent administration of estradiol (100 ppm) and atrazine (1000 ppm) in the diet decreased growth and feed intake 63.2 and 27.6%, respectively, versus control ($p < 0.05$). Addition of tamoxifen (an anti-estrogen) to the diet marginally inhibited the deleterious effects of estradiol on growth, feed intake, and liver-somatic index.

TABLE 4
Comparison of Atrazine and Pair Feeding on General Toxicity and Reproductive Development in Photostimulated Male Quail

Treatment	Average daily gain	Feed intake	Liver	Liver-somatic index	Testes weight	Gonadal-somatic index	Plasma testosterone (pg/ml)	Plasma estradiol (pg/ml)
Control	1.1 ± 0.17^{A}	15.4 ± 0.77	2.7 ± 0.16^{A}	2.1 ± 0.12	586 ± 44	0.5 ± 0.04	1105 ± 360	<15
Atrazine (1000 ppm)	0.4 ± 0.17^{B}	14.4 ± 0.77	2.1 ± 0.16^{A}	1.8 ± 0.12	484 ± 47	0.4 ± 0.04	691 ± 360	<15
Pair-fed[a]	0.1 ± 0.17^{B}	14.0 ± 0.77	2.0 ± 0.16^{B}	1.9 ± 0.12	474 ± 47	0.4 ± 0.04	671 ± 389	<15

Note. Data presented as least-squared means ± pooled SEM ($n = 7$ per group).
[a]Pair-fed birds received a daily amount of feed equivalent to the average intake of the atrazine-treated birds the previous day.
Unlike superscripts (A, B) are different at $p < 0.05$.

In study 7, the effects of atrazine on growth in the presence of systemically administered testosterone were examined. The results of this study are presented in Table 8. Addition of atrazine (1000 ppm) to the diet or the presence of a testosterone implant did not influence growth, feed intake, or liver weights versus control. However, concurrent administration of atrazine (1000 ppm) and systemic testosterone decreased growth 42.1% versus control ($p < 0.05$) (Table 8). No other parameters were affected by this combination.

Atrazine and the Hypothalamo-Pituitary-Gonadal Axis

The results of study 6 are outlined in Figure 2. Atrazine at 1000 ppm in the diet did not influence testes weight versus control. Administration of estradiol in the diet decreased testes weight 84.1% versus control ($p < 0.05$). Concurrent administration of atrazine and estradiol in the diet decreased testes weight 96.6% versus the negative control ($p < 0.05$). This decrease was observed to be 78.6% more than the effect of estradiol alone ($p < 0.05$). In contrast, addition of tamoxifen to an estradiol containing diet marginally inhibited the effect of estradiol on testes weight.

In study 7, the effects of atrazine as a putative anti-estrogen were further examined. These results are outlined in Figure 3.

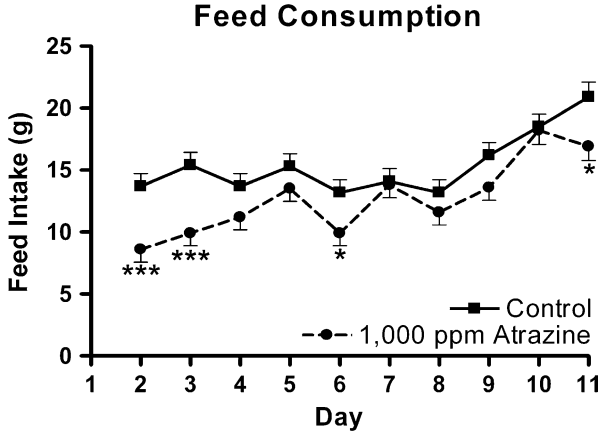

FIG. 1. Effect of dietary atrazine on feed intake in atrazine-treated male quail. Presented as least-squared means ± pooled SEM ($n = 5$–7 per group per day). Different from control group on same day, *$p < 0.05$, ***$p < 0.001$.

Atrazine at 1000 ppm in the diet had no effect on testes weight but reduced circulating concentrations of LH 35.7% versus control ($p < 0.05$). Addition of testosterone in the form of subdermal implants decreased testes weight and circulating concentrations of LH 91.8 and 69.1%, respectively, versus control. Administration of atrazine in the diet in the presence of testosterone decreased testes weights 95.8% versus control ($p < 0.05$). This decrease was observed to be 49.1% more than the effect of testosterone alone ($p < 0.05$). However, the combination of atrazine and testosterone had no additive effect on circulating concentrations of LH.

DISCUSSION

Atrazine is a triazine herbicide that inhibits photosynthesis in plants. It is widely used in the United States with up to 36,000 metric tons applied per year (USEPA, 2004). It has low acute toxicity to birds and mammals (Heath *et al.*, 1972; USEPA, 2002). However, atrazine has been reported to exhibit detrimental effects on reproduction in amphibians at very low concentrations (Hayes *et al.*, 2002, 2003) and in mammals at very high concentrations (e.g., Babic-Gojmerac *et al.*, 1989; Stoker *et al.*, 2000). There is little information on the effects of atrazine in birds, despite wild birds potentially being exposed to field applications of the herbicide during their seasonal reproduction.

Birds in the temperate zone undergo rapid sexual maturation due to photostimulation in the spring (Dawson *et al.*, 2001; Follett *et al.*, 1985). At this time, they may be particularly sensitive to the effects of putative toxicants. We have established a system to evaluate the effects of putative endocrine disruptors (including estrogens and anti-estrogens) during sexual maturation of male quail. While the Japanese quail is highly responsive to photoperiod, photoperiods of slightly less than 12 h of light are needed to induce gonadal development in the male quail (Follett and Maung, 1978). These effects have been observed both in a controlled setting and under natural photoperiods (Follett and Maung, 1978). While sustained concentrations of gonadotropins and steroid hormones vary, the initial surge of gonadotropin is similar at

TABLE 5
Effects of Systemically Administered Atrazine on Indices of General Toxicity and Reproductive Development in Photostimulated Male Quail

Treatment	Average daily gain	Feed intake	Liver	Liver-somatic index	Testes weight	Gonadal-somatic index	Left-to-right testis ratio	Circulating LH (ng/ml)
Subcutaneous injections								
Vehicle	1.2 ± 0.15	17.1 ± 0.76	2.6 ± 0.27	2.0 ± 0.17	939 ± 25	0.7 ± 0.19	1.2 ± 1.30	5.5 ± 1.56
1 mg/kg/day Atrazine	0.9 ± 0.15	15.6 ± 0.76	2.1 ± 0.27	1.8 ± 0.17	1075 ± 25	0.9 ± 0.19	1.1 ± 1.30	6.7 ± 1.68
10 mg/kg/day Atrazine	1.2 ± 0.15	16.1 ± 0.76	2.0 ± 0.30	1.9 ± 0.19	1118 ± 23	0.9 ± 0.18	1.3 ± 1.20	5.1 ± 1.56
1 mg/kg/day E_2-Benzoate	1.0 ± 0.15	15.7 ± 0.76	3.2 ± 0.27	2.6 ± 0.17	500 ± 25*	0.4 ± 0.19*	3.9 ± 1.30	N.D.
Silastic implant								
Cholesterol	1.3 ± 0.26	19.4 ± 1.13	2.7 ± 0.16	2.0 ± 0.14	727 ± 131	0.6 ± 0.10	1.2 ± 0.23	5.5 ± 1.20
Atrazine[a]	1.3 ± 0.28	18.2 ± 1.13	2.9 ± 0.16	2.2 ± 0.14	1064 ± 142	0.8 ± 0.11	1.1 ± 0.24	5.8 ± 1.29
E_2-Benzoate	0.9 ± 0.28	16.7 ± 1.13	3.9 ± 0.16***	3.0 ± 0.14***	171 ± 200*	0.1 ± 0.16*	1.5 ± 0.35	N.D.

Note. Data are presented as least-squared means ± pooled SEM ($n = 7$ per group).
[a]Implant delivered daily dose of ~1.42 mg/kg/day.
Different from control, *$p < 0.05$, ***$p < 0.001$; N.D. = not determined.

photoperiods ranging from 12 to 20 h of light (Follett and Maung, 1978). The combination of the photoperiodic threshold and these similarities in gonadotropic surge suggest our system is a reasonable model for a springtime effect.

Within our studies, an acclimation period to changes in daylength was not employed. This change in photoperiod may be hypothesized to influence the response of a bird to a toxicant, perhaps due to the rapid increase and activity of circulating concentrations of reproductive hormones. It is well documented that, at the threshold photoperiod, the Japanese quail exhibits a sharp increase in circulating concentrations of gonadotropin (e.g., Follett and Maung, 1978; Gledhill and Follett, 1976). These hormones induce the rapid development of the gonads, testes weight increasing logarithmically for the first half of development (Follett and Maung, 1978; Mather and Wilson, 1964). Furthermore, a slow, progressive increase in circulating concentrations of testosterone is observed; however, this does not parallel concentrations of LH (Follett and Maung, 1978). As the timing of environmental exposures to a toxicant such as atrazine cannot be controlled, the studies presented are useful to model this putative additive effect.

The results presented support the low general toxicity of atrazine. There was no effect of atrazine on mortality. Similar to the situation in rodents (Stoker *et al.*, 2000), atrazine at 1000

TABLE 6
Morphometric Analysis of the Microanatomy of the Left Testis in Atrazine-Treated Photostimulated Male Quail

Treatment	n	Class[a,b]	Lumen[c]	Seminiferous tubule diameter (μm)[d]	Diameter:testis weight
Control	34	2.6 ± 0.06	1.1 ± 0.07	164 ± 6	0.6 ± 0.02
Atrazine					
10 ppm	12	2.9 ± 0.12	1.1 ± 0.11	193 ± 12	0.4 ± 0.04*
100 ppm	19	2.8 ± 0.09	1.0 ± 0.09	177 ± 9	0.5 ± 0.03
1000 ppm	35	2.7 ± 0.07	1.1 ± 0.06	168 ± 6	0.5 ± 0.02
Pair-fed	7	2.8 ± 0.14	1.2 ± 0.14	140 ± 13	0.7 ± 0.04

Note. Data were pooled from studies 1, 2, 3, and 7 and are presented as least-squared means ± SEM.
[a]Ten seminiferous tubules were analyzed within each sample.
[b]Classification: 1: resting spermatogonia, 2: dividing spermatogonia with few spermatocytes, 3: many (>10) spermatocytes.
[c]Lumen classification: 0: no lumen, 1: lumen < 1/3 of total size, 2: lumen > 1/3 of total size.
[d]Tubule diameter measured using an ocular micrometer.
Different from control, *$p < 0.05$.

TABLE 7
Effect of Atrazine in the Presence or Absence of Estradiol on Indices of General Toxicity in the Photostimulated Male Quail

Treatment	Average daily gain (g)	Feed intake (g)	Liver (g)	Liver-somatic index
Control	1.9 ± 0.15^{A}	19.9 ± 0.49^{A}	3.3 ± 0.23^{A}	2.6 ± 0.15^{A}
1000 ppm Atrazine	1.3 ± 0.14^{B}	17.1 ± 0.45^{B}	2.9 ± 0.21^{AB}	2.5 ± 0.14^{A}
Tamoxifen[a]	1.8 ± 0.15^{A}	19.3 ± 0.57^{A}	3.1 ± 0.26^{A}	2.4 ± 0.18^{A}
Estradiol[b]	1.3 ± 0.16^{A}	16.8 ± 0.54^{B}	4.0 ± 0.25^{AC}	3.3 ± 0.17^{B}
Estradiol and tamoxifen[c]	1.7 ± 0.20^{A}	18.6 ± 0.66^{AB}	3.9 ± 0.31^{A}	3.0 ± 0.21^{AB}
Estradiol and atrazine[d]	0.7 ± 0.17^{B}	14.4 ± 0.57^{C}	3.1 ± 0.26^{A}	2.9 ± 0.18^{AB}

Note. Data presented as least-squared means ± pooled SEM ($n = 8$ per group).
[a]100 ppm tamoxifen.
[b]100 ppm 17β-estradiol.
[c]100 ppm estradiol and 100 ppm tamoxifen.
[d]100 ppm estradiol and 100 ppm atrazine.
Unlike superscripts (A, B, C) different at $p < 0.05$. For liver weight, atrazine and estradiol are different at $p < 0.05$; however, no other combinations differ.

TABLE 8
Effect of Atrazine in Presence of Implanted Testosterone on Indices of General Toxicity in Photostimulated Male Quail

Treatment	Average daily gain (g)	Feed (g)	Liver (g)	Liver-somatic index
Control	1.9 ± 0.18^{A}	19 ± 0.9	3.7 ± 0.36	2.9 ± 0.24
Atrazine (1000 ppm)	1.9 ± 0.21^{A}	20 ± 1.0	3.7 ± 0.41	2.8 ± 0.27
Testosterone	1.9 ± 0.18^{A}	23 ± 0.9	3.2 ± 0.36	2.5 ± 0.24
Testosterone + atrazine	1.1 ± 0.18^{B}	21 ± 0.9	3.0 ± 0.36	2.5 ± 0.24

Note. Values are presented as least-squared means ± pooled SEM ($n = 9$ per group).

Unlike superscripts (A, B) are different at $p < 0.05$.

ppm in the diet reduced feed intake and growth in male quail (Tables 1 and 4, Fig. 1). In one study, liver weights were affected by atrazine (Table 4); however, this effect was not different from that observed in pair-fed birds. Atrazine administered systemically (sc injections and Silastic implants) up to 10 mg/kg/day did not affect general toxicity (growth, feed intake, and liver weights) in the sexually maturing male bird (Table 5). These results suggest that the negative effects of atrazine on growth are minute and only seen at concentrations of at least 1000 ppm in the diet (~150 mg/kg/day). As demonstrated in the pair-feeding study (study 5), the effects can be partially attributed to energy restriction due to reduced feed intake, perhaps due to feed aversion.

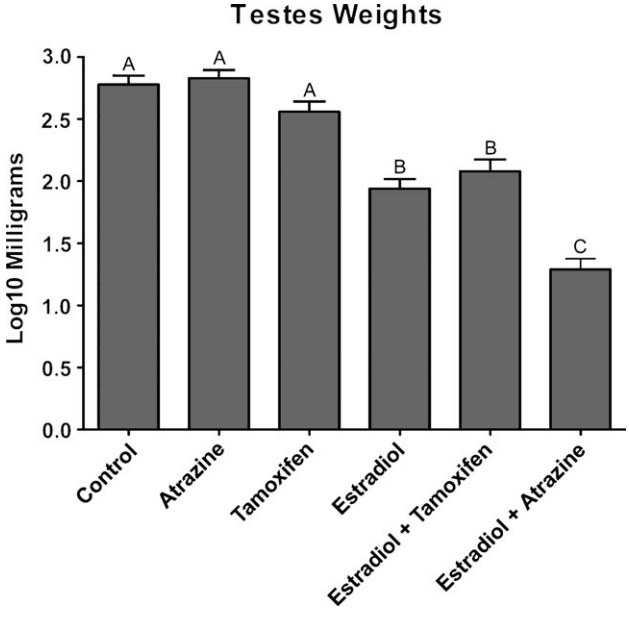

FIG. 2. Effect of atrazine and estradiol on testes weight during a photostimulated cycle of gonadal maturation in male quail. Treatments: estradiol: 100 ppm, atrazine: 1000 ppm, and tamoxifen: 100 ppm. Data are presented as least-squared means ± pooled SEM ($n = 8$ per treatment). Unlike superscripts (A, B, C) are different at $p < 0.05$.

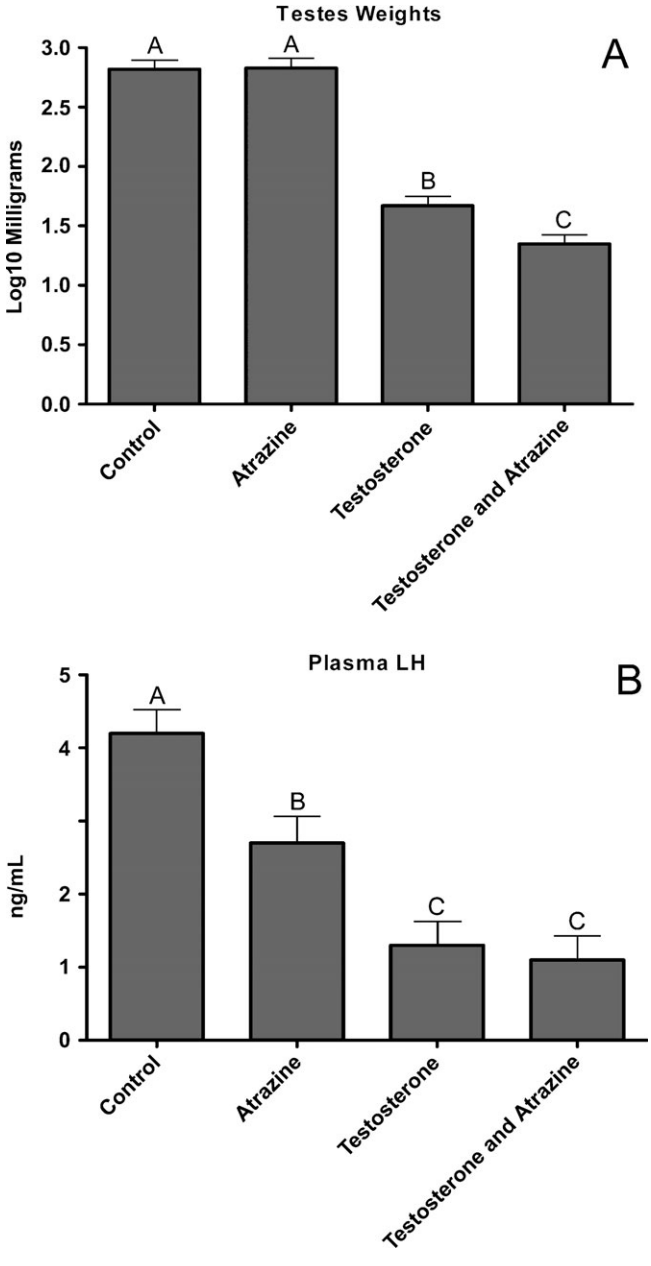

FIG. 3. Effects of atrazine and systemic testosterone on testes weight (A) and circulating concentrations of LH (B) in sexually maturing male quail. Data are presented as least-squared means ± pooled SEM ($n = 9$ per treatment). Unlike superscripts (A, B, C) are different at $p < 0.05$.

Atrazine exhibited an additive toxic effect on growth and feed intake with exogenous estradiol (Table 7). Furthermore in one study, atrazine alone had no effect on growth or feed intake but in the presence of testosterone decreased growth without a change in feed consumption (Table 8). Due to the presence of chemical mixtures in the environment, including natural and synthetic steroids, the present results suggest that caution should be exercised in interpreting the effects of atrazine in animal systems.

Based on previous reports in amphibians (Hayes *et al.*, 2002, 2003), it was hypothesized that atrazine would exhibit an estrogenic effect on the HPG axis in the sexually maturing male quail. In the male frog (*Xenopus laevis*), atrazine reduced circulating concentrations of testosterone (Hayes *et al.*, 2002). Furthermore, in the male rat, atrazine increased circulating concentrations of estradiol and decreased circulating concentrations of LH (Stoker *et al.*, 2000). In the present studies, atrazine exhibited only modest effects on the HPG axis of the sexually maturing bird. For example, at 10 ppm in the diet, atrazine increased circulating concentrations of estradiol in the male quail (Table 3). In male quail, circulating concentrations of estradiol are extremely low (e.g., Niemann *et al.*, 2004; Schumacher *et al.*, 1988; Watson *et al.*, 1990) and as such, the male may be expected to be highly sensitive to changes in concentrations of estrogen. However, in study 3, atrazine had no effect on plasma concentrations of estradiol (all samples and controls fell below the limit of detection for the assay) (Table 4). Thus, it is unclear if these changes in circulating concentrations of estradiol would be consistently observed in quail exposed to atrazine.

Male quail receiving atrazine in the diet at 1000 ppm exhibited an increase in circulating concentrations of testosterone (Table 3). This effect was inconsistently observed as concentrations were not different in study 3 (Table 4). In contrast, in the male rat and frog, atrazine decreased circulating concentrations of testosterone (Hayes *et al.*, 2002; Trentacoste *et al.*, 2001). In one instance, in the male quail, atrazine decreased circulating concentrations of LH (Fig. 3B)—an effect similar to that found in the rat (Stoker *et al.*, 2000). Atrazine increased testes weight and the gonadal-somatic index in quail receiving atrazine at 10 ppm in the diet (Table 1). Furthermore, atrazine at 10 ppm decreased the seminiferous tubule-to-testis weight ratio (Table 6). However, testes weights (Tables 2 and 4) and other morphometrics of the left testis (classification, lumen, and tubule diameter) were not different from control at any other concentration of atrazine tested (Table 6). Due to inconsistencies and modesty in effect, the physiological importance of the atrazine-induced changes in circulating hormone concentrations and testis development is tenuous.

In the Japanese quail, as predominantly in other birds, testosterone exerts a negative-feedback effect at the level of the hypothalamus following aromatization to estradiol (Davies *et al.*, 1980). These effects are reflected in gonadal weight and, as such, in the current system, testes weights were used as an indicator of FSH secretion and estrogenic/anti-estrogenic effects on the avian HPG axis. In the human and rat, atrazine has little affinity to bind the estrogen receptor and, conversely, may possess low levels of anti-estrogenicity (Connor *et al.*, 1996; Tennant *et al.*, 1994). Atrazine did not exhibit any estrogenic (Tables 2 and 4) or anti-estrogenic (Figs. 2 and 3A) activity on the testes in the male Japanese quail. Conversely, atrazine at 1000 ppm augmented the negative-feedback effect on the testes of both of the major sex steroids—estradiol and testosterone (Figs. 2 and 3A). While testes weights were not different between controls, atrazine-treated and pair-fed birds (Table 4), this relatively small change in testes weights (Figs. 2 and 3A) could be attributed to reduced feed intake. Regardless, the effect of atrazine on testes weights in the presence of sex steroids provides insight into the marked sensitivity of the avian HPG axis to the direct or indirect effects of a putative toxicant.

In the rat, atrazine inhibited the secretion of LH without influencing the function of the estrogen receptor (McMullin *et al.*, 2004). Furthermore, in the gilt and rat, atrazine and its metabolite diaminochlorotriazine (DACT) decreased the sensitivity of the LH surge to exogenous gonadotropin-releasing hormone (GnRH) (Gojmerac *et al.*, 2004; McMullin *et al.*, 2004). In the male quail, atrazine did not affect circulating concentrations of LH with the exception of one instance (Fig. 2B). The present evidence suggests that atrazine up to 1000 ppm in the diet has little influence on the secretion of LH.

In summary, the present results demonstrate that atrazine at concentrations at or above 10 ppm in the diet exhibit few and inconsistent effects on the reproductive system in the sexually maturing male Japanese quail. It is unlikely that the changes observed would have a profound effect on sexual development or reproduction of male birds in the wild.

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